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16. ABSTRACT

This study was designed to examine the relationship of waterborne enteroviruses to infections and disease. Young weanling swine and their homologous enteroviruses were chosen as the model system: The porcine digestive tract is like that of man, but pigs can be handled under more closely standardized conditions than humans or other primates. Known quantities of two enteroviruses were administered in 5 ml of drinking water in such a way that the subjects were obliged to swallow all of it. The intact animal was found to be about 1000 times (600 to 750 for one virus and 1800 to 2500 for the other) less likely than the tissue cultures to be infected by a given quantity of enterovirus. The ratio did not depend on whether the animals were fed just before challenge. The probability of infection was cumulative with iterated small doses: this indicated that there was, in the strict sense, no minimum infectious dose. None of the infected animals became ill, despite the reported virulence of the challenge viruses. Chlorine treatment of a concentrated virus suspension, which reduced infectivity to a level detectable by cytopathic effect but not plaque formation in tissue culture, left enough virus to infect one of five challenged subjects. Neither of two colostrum-deprived pigs, challenged by stomach tube with 20 plaque-forming units of enterovirus at 1½ hr of age, became infected.

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INFECTIVITY AND PATHOGENICITY OF ENTEROVIRUSES INGESTED WITH DRINKING WATER

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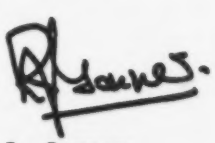
FOREWORD

The U. S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The primary mission of the Health Effects Research Laboratory in Cincinnati (HERL) is to provide a sound health effects data base in support of the regulatory activities of the EPA. To this end, HERL conducts a research program to identify, characterize, and quantitate harmful effects of pollutants that may result from exposure to chemical, physical, or biological agents found in the environment. In addition to valuable health information generated by these activities, new research techniques and methods are being developed that contribute to a better understanding of human biochemical and physiological functions, and how these functions are altered by low level insults.

This report provides an evaluation of the number of viral units required to initiate infection in an animal model. Some aspects of this study precluded the use of human subjects. Therefore, inferential judgments on the application of the data to man must be made.

Certain environmental exposures to viruses are subject to man's control and zero exposure from some sources is technically possible, e.g. drinking water and wastewater used for irrigation. However, treatment required to eliminate these potential sources of viral exposure is costly and may be economically unsound unless a significant health risk exists. Infective dose data is basic to determining the risk of viral infections from such environmental exposures. This work has provided a significant contribution to the data base needed to make sound risk judgments in this area.



R. J. Garner
Director
Health Effects Research Laboratory

PREFACE

Snow's epidemiologic studies on cholera in mid-19th-century London proved that disease could be transmitted through drinking water. Viruses and their ability to cause human disease were discovered near the turn of the century. Later, epidemiologic studies showed that some virus diseases, such as poliomyelitis and hepatitis A, were also transmissible through drinking water. Increasingly sensitive methods for detecting viruses (especially enteroviruses) in drinking water have been developed in recent years, but it is not yet clear whether the viruses that may thus be detected denote a threat to human health.

The present study looks at waterborne enteroviruses as potential causes of infection and disease. Swine and their homologous enteroviruses were chosen as the model system to produce the most information applicable to human health with the least cost and risk to research personnel. Devising the needed experimental procedures took much time and effort, since a study of this type and design appears to be unprecedented. Thus, in addition to the insights gained into the initiation of infections by waterborne enteroviruses, the study has produced a set of techniques applicable to research on the transmission of viruses and other infectious agents through water and, perhaps, via other environmental routes. We are grateful to those at the U.S. Environmental Protection Agency who were willing to risk funding an unconventional project. We believe that this report vindicates their judgment.

ABSTRACT

This study was designed to examine the relationship of waterborne enteroviruses to infections and disease. Young weanling swine and their homologous enteroviruses were chosen as the model system: The porcine digestive tract is like that of man, but pigs can be handled under more closely standardized conditions than humans or other primates. Porcine enteroviruses resemble those of man in every way, but they infect swine so specifically that handling the most virulent of the porcine agents is apparently no threat to the health of research personnel. Known quantities (as measured by the plaque technique in tissue cultures) of two enteroviruses were administered in 5 ml of drinking water in such a way that the subjects were obliged to swallow all of it. The host's body was found to be about 1000 times (600 to 750 for one virus and 1800 to 2500 for the other) less likely than the tissue cultures to be infected by a given quantity of enterovirus. The ratio did not depend on whether the animals were fed just before challenge. The probability of infection was cumulative with iterated small doses: this indicated that there was, in the strict sense, no minimum infectious dose. None of the infected animals became ill, despite the reported virulence of the challenge viruses. Chlorine treatment of a concentrated virus suspension, which reduced infectivity to a level detectable by cytopathic effect but not plaque formation in tissue culture, left enough virus to infect one of five challenged subjects. Neither of two colostrum-deprived pigs, challenged by stomach tube with 20 plaque-forming units of enterovirus at 1½ hr of age, became infected.

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SECTION 1

INTRODUCTION

Once a virus infection has begun, little can be done therapeutically to influence its outcome. Therefore it is important that virus infections, including those that might be transmitted via drinking water, be prevented when possible.

Viruses with a known potential for transmission via drinking water are those that emanate from the human intestinal tract. They are shed in feces, are relatively stable outside the host, and must be ingested to produce an infection. Although the host that produces the virus may not be perceptibly ill as a result of the infection, such viruses cannot be regarded as part of the normal intestinal flora--most persons in countries such as the United States do not generally harbor viruses in their intestines. Thus there is a finite limit to the amount of virus that might contaminate the environment by way of feces and sewage. Infection with an enteric virus usually results in durable immunity; insofar as people in the U.S. are relatively unlikely to have been infected previously with any given enteric virus, they are more susceptible than most of the world's population.

Some of these viruses are shed from the pharynx, as well as from the intestines in feces. The most common route by which enteric viruses are transmitted is directly from person to person, but contamination of water or food with feces or wastewater has led to transmission of intestinal virus infections on occasion. Whether the virus is transmitted by personal contact or through water or food, little is known of how infection begins in the human body. That is, neither the identity of the cells in the digestive tract with which ingested virus first interacts nor the precise mode of interaction between the virus and these cells has yet been determined.

In the present study, enteroviruses (the most numerous group of the human intestinal viruses) were selected for peroral administration in drinking water. The objects of the study were to learn how much virus had to be ingested to cause infection and whether a greater quantity was necessary to cause disease. Because the aim was to produce disease, virulent virus had to be used and human subjects were not appropriate. Instead, we chose to work with swine because their digestive tract is very similar to that of man and because their homologous enteroviruses resemble those of man in every way except for their host specificity.

Two serotypes of porcine enteroviruses were used in these experiments. Doses were administered perorally with drinking water to determine (a) how much of each serotype, measured in plaque-forming units (PFU), constituted an oral infectious dose (OID, or dose required to produce infection in the host)

and (b) whether the peroral pathogenic dose (OPD, or dose required to produce symptoms of disease in the host) was the same or larger. We also studied the effect of presence or absence of food in the stomach, of administration of the virus in repeated small doses, of disinfection of virus with chlorine, and of challenge of newborn pigs by gavage. Important insights into these questions were gained, even though not all of them were answered explicitly.

SECTION 2

CONCLUSIONS

A given quantity of enterovirus is more likely to produce an infection if inoculated into a susceptible tissue culture than if ingested by a potential host organism with drinking water. The OID for waterborne enterovirus ingested by susceptible young pigs was calculated with the aid of the Poisson formula to be approximately 1800 to 2500 PFU of porcine enterovirus type 3 (strain ECPO-6) and 600 to 750 PFU of type 7 (strain 05i). The PFU:OID ratio is a measure of the relative efficiency with which a virus infects a tissue culture and a whole host organism. It obviously varied with the virus type or strain, but it did not seem to depend on the host organism. Results of successive experiments with a given virus strain were comparable, even when groups of pigs were obtained from different herds. This result suggests that in the absence of detectable antibody against the virus, the infection of individuals at low doses of virus depends on chance rather than on individual differences in susceptibility. In a single experiment with animals that had received half their breakfast, instead of fasting for at least 12 hr before being challenged with waterborne virus, the proportion of animals that became infected was unchanged. Thus virus infectivity might be the same whether the virus is ingested in water with a meal or on an empty stomach, and perhaps the same even if the virus is present in the food rather than in the water. Even though both strains of virus used in this study were purportedly virulent, no perceptible illness resulted from the virus infections produced with dosages up to 10^4 OID.

The risk of becoming infected appeared to cumulate with ingestion of repeated, small doses of virus. This result indicates that the likelihood of infection can be predicted by simple probability statistics and that there is no absolute minimum dose that must be ingested at a single instance to produce infection.

Virus that had been disinfected by chlorine evidently was not reactivated after ingestion. Millions of PFU of enterovirus were reduced to a level of <1 PFU that was just detectable by cytopathic effect in tissue cultures. Only one of five pigs challenged with this chlorine-treated preparation became infected.

Two colostrum-deprived newborn pigs, challenged via stomach tube with 20 PFU of porcine enterovirus type 3 at 1½ hr of age and then reared with bovine colostrum in which no antibody against the virus could be detected, did not become infected. This contrasts with previously published reports that newborn humans became infected when challenged in this manner with as little as 1 TCD_{50} of vaccine poliovirus.

SECTION 3

RECOMMENDATIONS

The results of the present study indicate that enteroviruses infect their homologous host perorally with lower efficiency than they infect monolayer cell cultures of the same species. The risk of infection appeared to cumulate with ingestion of repeated small doses: this merits further research. The finding that enteroviruses infect tissue cultures with greater efficiency than they infect the intestines after ingestion with drinking water is surprising and merits further study. Nothing in the evolutionary history of enteroviruses should cause them to favor tissue cultures over the intestines as hosts. More should be learned about the barriers to virus infection that operate at the cell, tissue, and organism levels. The virus type that appears to be more efficient when peroral infectious doses are expressed as PFU might instead simply be found to be a very inefficient initiator of plaques if the number of virus particles inoculated were known in each instance. Therefore, the fate of virus particles in tissue cultures and in the intestines should be investigated.

Most intestinal virus infections fail to cause overt illness, as did all of the infections produced in the present study. Still, the possibility that a virus infection will cause disease is the ultimate reason for wanting to prevent transmission of viruses in drinking water. The success of the oral polio vaccine indicates that there is reasonable empiric knowledge of the virus-dependent aspects of pathogenesis. What was found lacking in the present study was knowledge of the host-dependent aspects of the disease process. Among other things, investigation is needed of the influence of age and of other past and present insults to the host organism on the probability that illness will result from an intestinal virus infection.

Finally, the infected intestines should be studied as a source of virus shed from the host organism. The duration of shedding and the quantity of virus produced, as well as its possible association with sloughed cells, other fecal solids, and coproantibody, need to be investigated. The influence of these factors on contact transmission and on transmission through food and water of virus produced by an infected host needs to be better understood if control measures are to be applied appropriately and in proportion to their relative significance to public health.

SECTION 4

MATERIALS AND METHODS

Many of the methods used in this investigation evolved significantly over the 3-year study period. If the method that finally resulted is wholly adequate to its task, the method may be regarded as one of the products of the study. However, this was not intended to be primarily a methodology study, so the methods described are ordinarily reported in their most representative form (that is, the version by which another investigator could most reasonably expect to reproduce the reported results). Suggestions for further improvement of methods are included where appropriate.

VIRUSES

Eight serologic types of porcine enteroviruses have been recognized by the World Health Organization's Programme on Comparative Virology (1). In surveys of sera obtained principally from swine at slaughter, we learned that porcine enterovirus type 3 (PE3) and later type 7 (PE7) did not occur with detectable frequency in the area of Madison, Wisconsin.

PE3 (strain ECPO-6) was supplied by Prof. Edward H. Bohl of the Ohio Agricultural Research and Development Center at Wooster as the first cell culture passage from the lumbar cord of a gnotobiotic pig that had paralysis of the rear legs. The pig had been infected with PE3 at the fourteenth cell culture passage level.

PE7 (strain 05i) was obtained from Dr. Louis Kasza, now of the Department of Pathology, Bureau of Foods, U.S. Food and Drug Administration. Like the PE3 strain that we used, this agent had been isolated from swine on farms in Ohio (2). The preparation that we received had been passed seven times in swine kidney cell cultures at the Department of Veterinary Pathology, Ohio State University, Columbus.

Poliovirus type 1 (PO1), strain CHAT, was used as an analog of the porcine enteroviruses. It was obtained from the American Type Culture Collection (3) and had been used as a model virus in our laboratory for several years.

CELL CULTURES

The porcine enteroviruses are specific for swine cells. Primary cell cultures (called PSK) were prepared from kidneys of (a) pig fetuses collected

at a local slaughterhouse, (b) young weanling pigs obtained along with those used in experiments, and (c) uninoculated control animals after experiments. Animals other than fetuses were killed by electrocution before the kidneys were collected. Cells were freed from minced and washed kidney cortices by batch treatment with 0.25% trypsin solution. Washed cells were suspended in Eagle's minimum essential medium (MEM) with nonessential amino acids and 10% fetal calf serum, and they were ordinarily seeded in 25-cm^2 plastic flasks. Secondary cultures were produced in some instances by dividing cells harvested with trypsin from confluent primary cultures.

We also used cultures from an established line of minipig kidney (MPK) cells obtained from t he American Type Culture Collection (3). Seed cell stock was propagated in 150-cm^2 plastic flasks or in plastic roller bottles. Virus tests were conducted with confluent monolayers in 25-cm^2 plastic flasks. HeLa cells, prepared in similar fashion, were used when the virus was P01.

Viral cytopathic effects (CPE) were detected by microscopic examination of cells maintained at 36° to 37°C with 5 ml per 25-cm^2 flask of MEM containing nonessential amino acids and 5% fetal calf serum. Plaque assay was done in 25-cm^2 flasks from which the medium had been discarded. The volume of inoculum was 0.5 ml per flask, and the adsorption period was 90 min at 36° to 37°C. The residual inoculum was decanted, and the cells were overlaid with 5 ml of MEM plus nonessential amino acids, 5% fetal calf serum, 0.02 mg/ml neutral red, and 0.7% BBL or Difco purified agar. Flasks in which the agar medium had solidified were incubated cell-side-up at 36° to 37°C and observed daily for plaque formation on the basis of the neutral red vital dye. On occasion, plaques were demonstrated or contrasted semipermanently by removing the agar overlay and staining the remaining cells with crystal violet in formaldehyde solution (345 ml deionized water, 4.25 g NaCl, 130 ml 95% ethanol, 2.5 g crystal violet, and 25 ml 37% formaldehyde solution).

SWINE

The animals used during these studies were obtained from two University of Wisconsin experimental farms and three commercial farms. At least one other commercial farm had been tried and eliminated as an animal source before this project began. The essential problem is that porcine enteroviruses are highly enzootic in our area; the same may be true for other porcine enteric viruses and opportunistic bacterial pathogens. Only heroic sanitation measures were able to minimize the incidence of these viruses. No farm was apparently free of porcine enteroviruses all of the time.

Rearing practices and general sanitation differed from farm to farm. In a well-run farrowing system, pigs would have access only to their own dam (who would not ordinarily be infected with enterovirus) and could be expected to remain virus-free during the first few days of their lives (4). At the University's Mandt Farm, which practiced rigorous sanitation and took extreme measures to avoid the introduction of infectious agents to the herd, pigs were often (but not always) virus-free when 3 to 3½ weeks old, the age at which we would have preferred to obtain them. The likelihood of obtaining virus-free pigs of that age from other farms was even smaller.

Feces from each animal after it arrived at our animal quarters were tested for enteroviruses. The time that elapsed before this testing (including at least one blind passage) was completed was often 2 weeks, which meant that ordinarily the animal's 3-day acclimation period, administration of the challenge virus dose, and most of the subsequent observation period were over before the results of the first tests were known. Of eight groups of animals obtained at the age of at least 3 weeks, five groups included enterovirus-infected animals. Obviously, this percentage was not good enough.

FOSTERING

We were able to borrow a unit in which to raise younger swine. The unit had been designed and built originally by the University's Department of Meat and Animal Science. It consisted of a box inside of which were a slatted, false floor, a thermostatically controlled ventilation system, and dispensing troughs for milk replacer. Access and observation were permitted by hinged acrylic panels at the top. We added meshwork partitions that subdivided the interior into 10 sections. Pigs were left with their dam after birth, to ensure that they got a full feed of colostrum, and were moved into the fostering unit in our animal quarters at age 2 to 8 days. Even with the colostrum for passive immunity, as well as the antibiotic and supportive therapy by our staff and by University veterinary personnel, diarrheal illness occurred frequently, and it was not always possible to keep the pigs healthy and normal (or at times, even alive) to the age of 3½ weeks.

Nevertheless, the fostering unit accomplished its intended task of removing the pigs from potential sources of enterovirus infection long enough to complete testing of the initial fecal specimens before the animals were challenged experimentally with enterovirus in water. No pig that was used experimentally after residence in the fostering unit was ever found later to have been infected with an enterovirus at the time of challenge. We usually obtained 10 animals at a time, in the expectation that at least eight animals would remain available by the date that challenge was scheduled. Any extra animals could be used as a source of kidneys for cell cultures.

EXPERIMENTAL HOUSING AND FEEDING

The animals were to be housed during the actual experimental period under conditions that would preclude introduction of viruses other than at the time of challenge and would prevent the transfer of virus from one animal to another. Control animals, which had not been challenged with virus, were included in each experiment in a way that we thought would maximize the likelihood of detecting any lapse in technique or in sequestration of the animals.

Four monkey cages were initially adapted in our own laboratory for use in housing pigs. The cages had been fabricated of stainless steel, part of which was expanded mesh joined by welding. To limit air intake, the sides and bottom were covered with acrylic and with tempered hardboard, and sealed to the metal frame and solid panels with gun caulking compound. The mesh top of the cage was covered with four thicknesses of Owens-Corning glass fiber filter

medium FM-004 (for a total thickness of approximately 5 cm) and sealed at the edges. An acrylic front panel with an exhaust duct connector was fitted to each cage by means of four toggle clasps. Room air was drawn through the glass fiber filter, into the cage, and then out through connector and flexible ducting to a blower on a manifold mounted on the exhaust duct leading from the animal room. Air that had been through a cage went directly out of the entire building; the intake filter system was comparable to that used (and recommended) by the University of Wisconsin (UW) Gnotobiote Laboratory. Food and water containers were located entirely inside the enclosure. A light was hung on the back of each cage so that it would shine in through an acrylic back panel and enable observation of the animal that was inside. These enclosures were all that we had during the first four experimental runs in this project. They represented a great improvement over some that had been made for us on a trial basis before the project, but they were still somewhat inconvenient to enter when the animals were fed and watered twice daily and when the litter pans were cleaned.

Soon after the project was funded, two racks of two enclosures each were ordered from Germfree Laboratories, Inc., of Miami, Florida. These were molded as one seamless piece, including top, bottom, sides, and back, of glass-fiber-reinforced polyester gel. An acrylic door was hinged to the front, and provision was made for sealing around its edges. Each unit had its own prefitted glass fiber intake and exhaust filters and an exhaust blower, which we subsequently connected by flexible ducting to the animal room's air exhaust duct. When the units were received, they were fitted for gnotobiotic operation but were not adapted to house any particular species of animal. Internal arrangements for containment, feeding, and watering of the animal; lighting; and litter removal were all made to our specifications by the sheet metal shop of the UW physical plant. These enclosures were first occupied 8 months after the project began. A few further modifications have since been made to them.

Pigs in the early runs were weighed when they arrived. Weight gains could be determined anytime until virus was administered and the individual enclosures had to be operated on a sealed basis. Food solids were apportioned at the rate of approximately 5% of the animal's weight per day, and water at a minimum of 10% per day (5). Initially, a standard breakfast comprised a premix of 50 g instant nonfat dry milk, 150 g dry instant oatmeal, 0.6 g iodized salt, and one crushed multiple vitamin tablet, to which were added 14 g peanut butter, 1 medium (∿150 g) banana, one medium hardboiled egg, and ∿250 ml water before serving. A standard supper was made up of 30 g peanut butter, 130 g cooked ground beef, 75 g instant nonfat dry milk, one medium (∿150 g) banana, and ∿250 ml of water. Additional water was available ad libitum. Later versions of the ration sometimes substituted pasteurized process cheese for ground beef and an apple for one of the bananas. Feeding was ordinarily done at approximately 8 a.m. and 4 p.m. each day.

The cages were numbered sequentially (1 through 4 for the adapted monkey cages and 5 through 8 for the germfree enclosures) and were always entered in numerical order. Only four enclosures were available during the first four experimental runs. Enclosure 4 was always the one that contained the control animal that had not been challenged with virus. When the number of enclosures

increased to eight, one control animal was always in enclosure 8, and another was placed in an enclosure whose number had been selected randomly for that experimental run only. This scheme was intended to afford maximum likelihood that any lapse of technique, either in disinfecting enclosures after the previous run or in twice-daily entries for feeding and watering, would be revealed. Sterile, disposable gloves were worn and discarded after each animal was fed, and those who did the feeding had at least a B.S. degree and experience in microbiology. Nevertheless, control animals were found to be infected on three different occasions. Two of these three animals were found to have virus in their 14-day, but not their 7-day, fecal specimens (counted from the day on which virus challenges had been administered). This result suggested that lapses had occurred during feeding rather than in disinfecting the enclosures. However, the animal in position 8, which should have been at greatest risk from this standpoint, was never the one involved.

CHALLENGE

Except where noted, challenge virus was administered in Madison tap water (5 ml per animal) that had been boiled previously to eliminate residual chlorine, as indicated by absence of detectable antiviral activity. In the first seven runs, the virus was simply thawed on the day of challenge and diluted to the desired level. Thereafter, in the course of dilution on the day of challenge, the virus was filtered through a Nuclepore polycarbonate filter of 50 nm porosity to remove or break up any aggregates that might be present. Previous testing of stock virus suspensions by filtration at 50 nm had not produced any decrease in plaque titer, so aggregation of the virus may not have been a problem under these conditions.

The level of virus in a dose was ordinarily determined on the basis of dilution from standard virus suspensions. The titers of the standard suspensions were verified periodically by the plaque technique. A formula provided by Sobsey (12) yielded an estimate of ±25% of the mean (e.g., 1000 ± 250 PFU) for the 95% confidence interval of these titers.

The animals in each group were challenged in numerical order. The control pigs received 5 ml of water to which virus had not been added. Each animal was upended and the head tilted backward. The 5-ml dose was delivered at the back of the tongue, and the mouth was held shut so that the animal was obliged to swallow all of it. The enclosure was "sealed" after the animal was returned to it; thereafter, the inside of the isolator was under negative pressure with respect to the room, and all air was taken in through filter, except during feeding, watering, and cleaning.

OBSERVATION

The condition of the animals and whether they had eaten their food was ordinarily noted at each feeding. A blood sample was collected from the anterior vena cava (5) either when the animal was put into its isolator at approximately 3½ weeks of age or before challenge 3 days later. Serum from this sample was used to test for pre-existing passive immunity to the challenge

virus. Fecal samples were ordinarily collected before challenge and on days 7 and 14 after challenge. Another blood sample was collected on the last day of the run (ordinarily day 14).

We wanted to determine whether the animals became infected by the virus that was administered and whether illness resulted from the infection. The criteria for infection, assuming the absence of virus and of antiviral serum antibody from before-challenge samples, were to be the presence of virus in the stool sample from day 7 or 14, or the presence of serum antibody by day 14. As will be discussed below, the virus criterion proved more useful than the antibody criterion. The criteria for disease were to be signs of central nervous system involvement (including incoordination, paralysis, or death) or a rectal temperature in excess of 40.5°C. No animals met these criteria following virus challenge during the entire course of this study.

We were most anxious that neither of our model virus serotypes be introduced to the on-farm swine population in our area: Whatever might have been the consequences to animal health, introduction of these viruses would have further complicated procurement of experimental animals. For this reason, animals from all runs were presumed infected and were killed by electrocution after the observation period ended. Useful portions of the body were sometimes collected (e.g., kidneys from control animals for tissue culture), and the remainder was bagged and frozen for later incineration by the UW research animal resources facility.

TESTING FECAL SAMPLES

Fecal samples were collected from the isolators and frozen at -20°C to await testing. It was important to obtain samples that were as freshly voided as possible because feces dried rapidly in the litter pans because of the constant flow of air through the isolators. Drying, to the extent that it did occur in these experiments, probably did not affect virus infectivity; but it sometimes did complicate suspension of the fecal mass in diluent.

Feces (1 to 2 g) were placed in a disposable plastic (styrene) centrifuge tube, and sufficient phosphate-buffered saline (PBS), to which 2% fetal calf serum had been added, was placed in the tube to bring the total volume to 10 ml. The tube was agitated repeatedly with a Vortex mixer and refrigerated (~4°C) to allow the fecal mass to soften. Enough 0.1 N NaOH was added to bring the pH to 8.8 to 9 (sometimes 0.1 N HCl to pH 3.5, depending on whether coproantibody or adsorption to solids seemed of greater concern), and the fecal mass was broken up with a sterile tongue depressor and agitated several times with the Vortex mixer. Tubes were then placed in icewater in a sonic cleaning bath and treated at 20 kHz for 15 min. Fecal solids were sedimented by 30 min of centrifugation at 16,000 rpm. The supernatant fluid was adjusted to pH 7 and passed through Gelman cellulose triacetate filters of 0.45 and 0.20 μm nominal porosity (the latter was sterile). Confluent swine cell cultures (25 cm^2) were inoculated with 0.5 ml or 0.1 ml (two of each) of the filtered extract. If CPE were not seen after these cultures had been incubated at 36° to 37°C until the uninoculated control cultures deteriorated, the cultures were frozen and thawed twice, sometimes filtered at 0.20 μm

nominal porosity, and inoculated into additional cultures at 0.5 ml per flask.

SEROLOGY

As stated earlier, blood for serological testing was ordinarily drawn from the pig's anterior vena cava. The blood was allowed to clot for 1 hr at room temperature and held in the refrigerator for approximately 24 hr more. The serum was aspirated from the retracted clot and centrifuged for 30 min at 2500 rpm. The supernatant serum from this step was frozen at -20°C in small volumes for later testing.

The basic test for antibody against virus was plaque-reduction neutralization. Serum samples were ordinarily tested at dilutions of 1:20, 1:40, and 1:80 in PBS. Virus was diluted to an estimated 100 PFU/ml in PBS with 2% fetal calf serum. Sometimes serum was diluted 1:50 and a constant serum-varying virus procedure was used instead. In either case, equal volumes of diluted serum and virus were mixed and incubated at 37°C for 1 hr in a water bath. Then the mixtures were inoculated at 0.5 ml per culture into duplicate 25-cm^2 cultures and tested by the plaque technique, which was described above. Sometimes the virus/serum mixtures were tested instead for their ability to cause CPE in cell cultures maintained with fluid medium.

SECTION 5

EXPERIMENTAL PROCEDURES

PERORAL INFECTIVITY EXPERIMENTS

The general purpose of many of the experiments was to determine the OID for virus in drinking water. The quantity of virus administered to all animals in a given experimental run was the same. Runs I, II, III, IV, V, VI, and VIII were intended primarily to determine the OID of PE3 (strain ECPO-6). The animals were challenged with 1, 10, 100, 1000, 1000, 250, and 250 PFU of PE3, respectively, in this series of experimental runs. Runs IX, X, XI, and XV were intended to determine the OID of PE7 (strain 05i); doses were 350, 1000, 250, and 250 PFU per animal, respectively.

PERORAL PATHOGENICITY EXPERIMENTS

The second stated objective of this study was to determine the quantity of virus that had to be ingested with drinking water to cause disease (the OPD). Experiments intended to reveal the OPD included run VII for PE3 and run XIII for PE7. The dose of PE3 per animal was 2.5×10^7 PFU of virus that had been extracted from the brain of a pig inoculated intracerebrally with the virus in an attempt to enhance its virulence. Because we had great difficulty producing useful quantities of either virus by extraction from brains of intracerebrally inoculated pigs (2,6), the PE7 virus administered in run XIII was an extract (supernatant fluid after 30 min of centrifugation at 13000 rpm, filtered through a sterile, 0.2-μm porosity, cellulose triacetate membrane) of a 10% suspension of fourteenth-day feces from run XI. Each of the challenged animals received approximately 10^5 PFU of PE7.

EMPTY VERSUS FULL STOMACH

In nearly all experiments conducted during this study, the animals were deprived of food for approximately 16 hr before the virus dose was administered. This empty-stomach condition might have influenced the efficiency with which virus ingested with drinking water would initiate infection, although it was impossible to predict whether the viurs would be helped or hindered. Furthermore, the empty-stomach condition would not necessarily be typical of a human that might ingest virus with drinking water.

This question was addressed in experimental run XII. Eight 30-day-old pigs were fed approximately half their breakfasts (37.5 g dry milk, 10 ml peanut oil, 57 g cheese, and 500 ml water). When this amount had been consumed, six of the animals were challenged with 5 ml of water containing 1000 PFU of PE3; the two control animals received 5 ml of virus-free water. The animals were each fed 37.5 g dry milk, 10 ml peanut oil, 1 egg, and 500 ml of water upon being returned to their isolators.

ITERATED DOSES

The most frequently used test dose of PE3, 1000 PFU, produced infections in about one-third of the animals challenged, whereas no infections were shown to result from a single dose of 250 PFU of PE3. This result suggests that there might be some level of virus below which infection cannot take place (a minimum infectious dose; 7). On the other hand, a corollary to the assumption that these viruses showed a Poisson distribution was that the risk of infection should cumulate with iterated doses. This question is exceedingly important from the standpoint of public policy. The matter was examined in experimental runs XIV and XVI.

The challenge protocol was the same for both runs, but the numbers of animals differed. Because of supply problems, only six animals were available for run XIV. At that time, it was uncertain that an infection could result from ingestion of as few as 250 PFU of PE3, so it seemed more important to expose as many animals to this dose as possible, at the expense of including the usual two controls. Five of the animals were challenged, and only one served as a control. On each of four successive days, each of the five animals received 250 PFU of PE3 in 5 ml of water, and the control pig received only the water. Once it was established that infection could result from repeated doses of this size, we scheduled run XVI as a full-scale experiment to confirm this finding. Six of eight animals received the 250 PFU of PE3 in 5 ml of drinking water on four successive days, whereas the two control animals received only the 5 ml of water on each of those days.

CHLORINE DISINFECTION

Chlorine is the disinfectant against which the efficiency of others is measured. Nevertheless, there has sometimes been concern that inactivation, as it is demonstrated in tissue culture, might be insufficient to prevent infection when the virus is ingested by a complex organism such as the human body. Run XVII examined this question.

PE3 (1 ml containing 3.6×10^7 PFU) was treated in a sonic bath, passed through a 50-nm porosity polycarbonate filter membrane, and added to 75 ml of 0.2% Clorox solution. This amounted to an applied dose of 50 mg of chlorine per liter in unbuffered solution at room temperature. The mixture was stirred for 5 min, and to it was added 24 ml of 0.1 M $Na_2S_2O_3$. This was stirred for an additional 10 min and served as the challenge virus suspension for run XVII. Only seven pigs were available for this experiment, so five were challenged, and the other two served as controls and received water only. The

dose administered (5 ml of the chlorine-treated suspension) contained what would have been 1.8×10^6 PFU of PE3 in the absence of chemical inactivation. Repeated testing of the portion that had not been administered to the pigs revealed some residual infectivity detectable without dilution, but not at 10^{-1}. Titration of the residual virus by the plaque technique was not successful.

GAVAGE CHALLENGE OF NEWBORNS

Plotkin and Katz (8) reported having produced infections in newborn humans with as little as 1 TCD_{50} of vaccine poliovirus administered by gavage. We attempted to duplicate this experiment, except that newborn pigs served as subjects.

Given the difficulty that we had experienced in fostering pigs that had been weaned after as few as 2 days with the sow, there would have been no reasonable prospect of success in experimenting with newborn animals had it not been for the reported finding of Prof. Jan Rapacz (UW Department of Meat and Animal Science) that newborn pigs could be reared entirely on cow colostrum (9). We obtained surplus bovine colostrum from the UW Department of Dairy Science and stored it frozen while determining that it had no antibody against PE3. We also had the four newer isolators modified by installation of a small infrared lamp in each. This modification enabled the animals to move about and find a place where the temperature suited them. Because of the amount of individual handling that was required, run XVIII included only four pigs, two of which were challenged with virus by stomach tube (8). The inoculum consisted of 2.5 ml of sterile deionized water (to which 2% fetal calf serum had been added in an attempt to limit losses of virus by sorption to equipment surfaces) containing a total of 20 PFU of PE3. The animals were collected as they were born, without allowing them to nurse or to ingest anything. Challenge took place about 1½ hr after the pigs were born. The first feeding of bovine colostrum was attempted 30 min later. Although newborn pigs are supposed to be able to drink from a dish, ours did not do well at it and were bottle-fed instead every 2 hr. Some diarrhea occurred in all four animals during the 2-wk observation period. The controls had a few more problems than the challenged animals, but all survived and were in apparently good condition when the experimental run ended.

SECTION 6

RESULTS

The results of experimental runs reported here are divided according to the kinds of information they were intended to produce. The Roman numeral designations indicate the true chronological sequence in which the experiments were done. Given the innovative nature of this study, what we learned from each of the experiments could not always be predicted on the basis of the stated purpose of the experiment.

PERORAL INFECTIVITY EXPERIMENTS

Run I

Four pigs were obtained from the UW Arlington Experimental Farm. The project officially began on November 1, 1975. These animals were born on September 5 and 6, 1975, and were among the last of the Arlington Farm's fall pigs. Because they were more than 6 weeks old by the time we received them on November 20, the pigs were larger than we wanted; but no others were available at the time. We did not have a scale on which animals of their size could be weighed. Blood samples were taken while the pigs were still at the farm; a definitive test for antibody against PE3 was not accomplished. Stool samples taken from all of the animals before challenge were shown to contain virus, as did those taken on days 7 and 14 after challenge. Because of the lapse of time involved in detecting the preexisting infection, we did not know about it in time to avoid doing the challenge. The challenge dose for run I was 1 PFU per animal, given on November 24. Although this run told us nothing about the effect of that size dose, we decided to increase the dose for the next run.

Run II

The pigs were born at the Arlington Farm on December 9, 1975 and received at our Institute December 30 at precisely 3 weeks of age. Their weights ranged from 5.15 to 6.95 kg. They were challenged on January 2, 1976, with 10 PFU of PE3, which was administered to the animals in isolators 1, 2, and 3. The pig in unit 4 served as the control. Before-challenge stool samples from pigs 2, 3, and 4 were later shown to contain virus; the apparent absence of virus from the stool of pig 1 may well have been due to a false negative test result. Virus was present in fecal samples from all animals on days 7 and 14 after challenge. Because we had no antiserum with which to identify PE3 at that time, all virus was assumed to be wild-type from the Arlington Farm. Again, definitive tests for antibody in the blood

serum were not accomplished. This run revealed nothing about the 10-PFU challenge dose. However, it did lead us to conclude that we would have difficulty getting virus-free pigs from the Arlington Farm, so no further animals were obtained from that source.

Run III

The four pigs were born at the UW Mandt Farm on February 20, 1976. They arrived at our facility on March 13 and ranged in weight from 5.5 to 8 kg. Three of the pigs were challenged on March 15 with 100 PFU of PE3. No virus was detected in the before-challenge stool samples or in those taken on days 7 and 14 after challenge. No antibody to PE3 was detected in blood serum from before challenge and from day 14 after challenge. This was the first experimental run that produced a clear result: We concluded that the OID was greater than 100 PFU for PE3.

Run IV

The four pigs were born at the Mandt Farm on March 22, 1976, and were received at our facility on April 12. They weighed from 7.35 to 9.1 kg. The challenge dose of 1000 PFU of PE3 was given to animals 1, 2, and 3 on April 14. These animals were found to have been infected with a virus that required repeated passage in cell culture for positive detection. Virus was eventually confirmed in before-challenge stool samples from animals 1, 2, and 4, in day-7 (after challenge) stool specimens from animals 2 and 4, and in day-14 stool specimens from animals 3 and 4. Very possibly all four animals were infected with this unknown virus throughout the run. Antibody against PE3 was absent from the before-challenge and day-14 blood samples. On day 14 (April 28), we thought there was reason to believe that pig 3 had been infected by the challenge dose. The other three pigs were sacrificed on that day, and pig 3 was given $\sim 10^6$ PFU of PE3 intraperitoneally and the same amount of virus per os in an effort to evoke a high titer of antibody against PE3 in this animal. Blood drawn on day 28 appeared to contain antibody against PE3, but because of the possible preexisting infection, this could not be assumed to be a source of monospecific antiserum for virus identification.

Run V

This was the first run in which we could accommodate eight pigs. They were born on the Mandt Farm on June 11, 1976, and arrived at our facility on July 1. Their weights, when they arrived, ranged from 4 to 7 kg. The animals in enclosures 4 and 8 served as controls; on July 5, the other six pigs were challenged with 1000 PFU each of PE3. Serum antibody against PE3 was absent at challenge, as was any virus in the feces of these pigs. Two of the challenged animals (numbers 5 and 7) were shedding virus on day 7, and virus was detected in the feces of pig 7 on day 14 after challenge as well. Results of tests for antibody against PE3 in the day-14 blood were equivocal. Run V, then, was the first that began with all animals uninfected and produced some clear-cut infections as a result of the challenge. Two of the six animals that had received 1000 PFU of PE3 were infected, whereas 100 PFU of the same virus had not infected any of the three pigs challenged in run III. A simple formula for estimating the OID is that given by Sobsey (12) for

estimating a most probable number of virus from a data set such as this. It derives from the Poisson formula and is written: $m = -\ln(q/n)$, which is to say that the most probable number of OID's present is the negative Napierian logarithm of the ratio between the number of negative results (q) and the number of animals inoculated (n). In this instance, the ratio of 4 animals uninfected among 6 inoculated indicates that the 1000 PFU dose was equivalent to 0.4 OID and, therefore, that the OID is approximately 2500 PFU of PE3. The next experiment was to be done with 0.1 OID, or 250 PFU of PE3 per animal.

Run VI

The eight pigs were born at the Mandt Farm on September 14, 1976. They weighed from 5.7 to 6.9 kg when we received them on October 5. The animals in isolators 6 and 8 served as controls in this run. The other six pigs were challenged on October 8 with 250 PFU of PE3 each. By agreement with the project officer, these animals were to be observed for 4 weeks after challenge. Unfortunately, virus was found to be present in feces collected from all eight pigs 2 days before challenge. Although a few of the intermediate samples did not contain detectable virus, feces from all eight animals had virus at the end of the 4-week period. We did not then have monospecific antiserum with which to determine whether any of this end-of-run virus was PE3. Obviously, the experiment needed to be repeated.

Run VIII

The eight pigs were the first obtained from a commercial source: They came from the Kaltenberg Farm near Waunakee, Wisconsin. Four of them had been born on January 24, 1977, and the other four on January 27. They were 4 and 7 days old, respectively, when we received them on January 31. Their weights were not recorded. They were held in the four newer, larger isolators (two pigs per unit) until challenge on February 24, when they were separated into all eight units. The pigs in units 3 and 8 were used as controls, and the other six received 250 PFU of PE3. Fecal specimens taken 5 days before challenge, as well as those taken weekly for 4 weeks after challenge, were negative for virus. Though the evidence that this series of runs supplies is a limited basis for the estimation of an OID for PE3, runs XII, XIV, and XVI, described in the following sections, are also pertinent to this question.

Run IX

This was the first effort to determine the peroral infectious dose for PE7. Eight pigs born on April 5, 1977, at the Mandt Farm were received on May 2 at 4 weeks of age. Weights were not recorded. The pigs in enclosures 4 and 8 were used as controls, and the remaining animals were challenged on May 6 with an estimated 350 PFU of PE7 (there were problems with the assay of this particular preparation). Three animals were later shown to have been infected at the time of challenge and to have remained so during the following 2 weeks: virus was present in the feces of animals 1, 2, and 5 on the challenge day and on days 7 and 14 thereafter. These three pigs apparently had been infected before they left the Mandt Farm. None of the other pigs became infected as a result of the experimental dose. This was the last

experiment in which we attempted to obtain animals 3 or more weeks of age and challenge them within a few days after they arrived at our facility.

Run X

Eight pigs born on August 19 and 20, 1977 at the Kaltenberg Farm were received on August 22 and placed in the fostering unit. Various health problems occurred during fostering, culminating in the death of one of the animals. At least those still alive on the challenge day (September 15) were without virus. The control animals for this run were in isolators 7 and 8, and isolator 3 was vacant. Pigs 1, 2, 4, 5, and 6 received 1000 PFU of PE7. Virus was found in the feces of each of these five animals on days 7 and 14 after challenge. This instance was one of the very few in which definitive immunologic responses could be demonstrated: None of the seven pigs had detectable serum antibody before challenge, and all of the five animals that were shown to have been infected on the basis of virus in their feces were also found by day 14 to have produced antibody that reacted strongly in the plaque-reduction neutralization test. Two of these animals received another 10^7 PFU of PE7 intramuscularly on day 14 and were bled from the heart on October 18 as sources of typing antiserum for this virus.

Run XI

The eight animals were born at the Mandt Farm on October 5, 1977, and received on October 7. These animals prospered in the fostering unit. They were transferred to the isolators, and those in units 7 and 8 were selected as controls. The others received 250 PFU of PE7 on October 28, at which time none was evidently infected. On day 7 after challenge, virus was detected in the feces of pigs 3 and 6, and on day 14, of pigs 3, 5, 6, and 7 (the latter was a control). The neutralization test results were anomalous. It seems likely that at least pigs 3 and 6 were infected by the challenge dose, that pig 7 was infected as a result of cross-contamination during the second week, and that pig 5 may have been infected in either way.

Run XV

The pigs were born on April 14, 1978, at the Mandt Farm. All of 10 animals survived the fostering period, which began April 19, but only eight were used (one per isolator). The animals in units 4 and 8 served as controls; the others were challenged with 250 PFU of PE7 on May 17. No virus was detected in the pre-challenge stools. Virus was present in the fecal samples on day 7 from pigs 6 and 7 and on day 14 from pig 4 (a control) and pig 7. The problem of cross-contamination leading to the infection of an uninoculated control animal was not encountered in runs XII, XIII, or XIV or in any subsequent run, although there had been a similar occurrence in run VII, which is described below. Two of the six animals challenged with 250 PFU of PE7 seem certainly to have been infected as a result. The results of this run and the others in this series indicate that the OID of PE7 is approximately 600 to 750 PFU.

PERORAL PATHOGENICITY EXPERIMENTS

Experimental runs VII and XIII were designed explicitly to induce disease in the pigs. Specific means of enhancing viral pathogenicity were tested, inasmuch as mere infection with the waterborne virus had not resulted in illness. The histories of the two model viruses indicated that they might produce central nervous system symptoms.

Run VII

The eight pigs were born on the Mandt Farm on October 7, 1976, and were received at our facility on November 8 at the age of 4½ weeks. The inoculum was derived from the cerebellum and medulla of a colostrum-deprived pig that had been inoculated intracerebrally by Thomas Yuill of the UW Department of Veterinary Science at 1 day of age and sacrificed at 7 days of age. The tissue suspension was treated with chloroform, with sodium dodecyl sulfate, and with ultrasound, then clarified by passage through a 5-μm porosity cellulose triacetate filter and purified on an Amicon PM30 ultrafilter membrane. Titrations of this preparation in MPK and PSK cell cultures yielded estimates of 4.7×10^6 and 5.0×10^6 PFU/ml, respectively. Thus the animals challenged with 5 ml of this preparation on November 10 received approximately 2.5×10^7 PFU, which is 10,000 times our estimate of the OID for PE3. None of the pigs was shedding virus in its feces before challenge. The animals in enclosures 5 and 8 served as the controls in this run. On day 7 and again on day 14 after challenge, virus was present in the feces of all six challenged animals and in that of control 5. This run (VII) was the first instance in which a supposedly uninoculated control became infected, and the only instance in which the infection was detected as early as day 7. Given the high level of virus used in this experiment, it seems possible that this animal was exposed while it was receiving its sham challenge, rather than as a result of cross contamination during its stay in the isolator. More important, some diarrhea and vomiting were seen on occasion in some of these animals during the 2-week observation period, but no signs were observed that were specifically indicative of central nervous system involvement.

Run XIII

Healthy, virus-free pigs were very difficult to obtain in January and February of 1978. Eleven 2- to 3-day-old pigs were obtained on January 25 from a commercial (Noltner) farm. By February 9, all but three of these had died in the fostering unit. The three survivors were moved to temporary quarters, and on February 10, 10 2-day-old pigs were received from another commercial (Double H) farm. By March 7, all but two of these had died in the fostering unit. These five survivors made up the group. The older animals were housed in isolators 1, 2, and 4, and the younger two were put into units 3 and 8 (the latter was the uninoculated control). The challenge preparation of PE7 was extracted from day-14 feces of run XI as described above. This procedure was chosen because we wanted to use virus passed directly from animal to animal, without intervening cell culture passages, and we had been unable to produce a satisfactory preparation of PE7 by intracerebral inoculation and extraction of the brain. The titer of the fecal extract was approximately 10^5 PFU per ml, and each of the four challenged animals received

5 ml of this, which is at least 3000 times the OID for PE7. None of the animals was shedding virus in its feces when challenged. All four challenged animals (but not the control) shed virus on days 7 and 14 thereafter. No significant illness was noted in any of these animals during the 2-week observation period, despite the large quantity of virus that had been administered.

EMPTY VERSUS FULL STOMACH

Run XII

The eight pigs were born on the Kaltenberg Farm on December 4, 1977. Of 10 animals received on December 7, one died by December 11, and the other nine grew well until the challenge day, January 3, 1978. The animals in isolators 2 and 8 were the controls for this experiment. The specifics of the challenge are described in the previous section. The challenge dose consisted of 1000 PFU of PE3 in 5 ml of water, administered between halves of the pigs' breakfast on January 3. No virus was found in the preinoculation fecal specimens from any of these pigs; virus was present in the feces of pig 5 on post-challenge day 7 and of pigs 3 and 5 on post-challenge day 14. This finding of infection in two of six pigs challenged when they had food in their stomachs is identical to that obtained in run V, when 1000 PFU of PE3 was administered to six pigs with empty stomachs.

ITERATED DOSES

Run XIV

The animal health problems mentioned earlier continued into March 1978. Ten pigs born March 20 and 21 on the Mandt Farm were received on March 22 and had all died in the fostering unit or been sacrificed moribund by March 27. The fostering unit was disinfected and refilled with 10 pigs (born March 26 at Mandt) on March 28. Six of these survived. The challenge procedure was as described in the preceding section. The pig in unit 8 served as a control, and the other five received 250 PFU of PE3 per animal on April 24, 25, 26, and 27. No virus was detected in the prechallenge fecal specimens. Post-challenge days 7 and 14 were numbered from the last of the four daily dosings. Virus was found in the 7-day samples from animals 1 and 7 and in the 14-day sample from pig 7. Thus two of five animals challenged with iterated doses of 250 PFU of PE3 became infected, whereas, none of six animals challenged once with 250 PFU of PE3 had been infected in experimental run VIII.

Run XVI

The animals were born at the Kaltenberg Farm on July 9 and 10, 1978. Eleven animals were received in the fostering unit on July 17. All of them survived until the time of challenge, although only eight could be used. The animals in enclosures 5 and 8 served as controls, and the other pigs were challenged in the same manner as in run XIV on August 7, 8, 9, and 10. None of the pigs was shedding virus before challenge. On day 7 after the last

dose, there was virus in the feces of animals 1, 4, and 6; these and pig 7 were found to be shedding virus on day 14. Clearly, iteration of small virus doses can result in infections.

CHLORINE DISINFECTION

Run XVII

Twelve pigs born on August 1, 4, and 5, 1978, were received on August 9. Five of these died during the period in the fostering unit, but the seven remaining were sufficient to do almost a full-scale experiment. Preparation of the chlorine-treated PE3 suspension is described in the preceding section. In the absence of chlorine treatment, the dose per animal would have been 1.8×10^6 PFU. The suspension was administered to the pigs in all units but 1 and 8 (which were occupied by the controls) and 5 (which was vacant). None of the animals was shedding virus at the time of challenge, but virus was found in the feces of pig 3 on days 7 and 14 thereafter. A trace of residual infectivity was detected in the undiluted inoculum by CPE, but not by plaque formation, in cell culture. This was the only instance in which the sensitivity of the whole organism seemed to approach that of cell cultures.

GAVAGE CHALLENGE OF NEWBORNS

Run XVIII

The four pigs were born at approximately 10 a.m. on October 2, 1978, at the Kaltenberg Farm. They were brought immediately to our facility, without nursing, and two of them were dosed by stomach tube with 20 PFU of PE3 at 11:30 a.m. These were placed in enclosures 5 and 7, and the sham-inoculated controls were housed in units 6 and 8. The animals were reared principally on cow colostrum, as described in the previous section. There were no pre-challenge fecal specimens, and no virus was detected in the samples collected on days 7 and 14. If newborns are inordinately sensitive to challenge by gavage, this admittedly limited experiment did not show it.

SECTION 7

DISCUSSION

The goal of this study was to produce information that would help to assess the significance of viruses in drinking water as a threat to human health. It has been established epidemiologically that viruses are occasionally transmitted through drinking water: Reported outbreaks of illness associated with public water supplies have been due to "breaks" in treating water derived from known contaminated sources or to contamination of finished water during distribution (10). Nevertheless, there has been manifest concern that viruses could be transmitted through drinking water and cause waterborne disease that could go unrecognized and unreported (11). Therefore, it is most unfortunate that studies done years ago concerning peroral infection by the polioviruses were discontinued once the procedures for administering the oral polio vaccine were established. Many questions significant to environmental virus transmission were left unanswered.

The major objectives of the study were to determine the OID for enteroviruses ingested with drinking water and to determine whether the OPD was greater. Some corollaries to these objectives were also included by agreement with the project officer. Because we had not developed the capability to enumerate virus particles, virus doses were expressed in PFU measured in monolayer cell cultures. Host responses to the challenges would be quantal (infection or none, disease or none), so the Poisson distribution that is used in most probable number (MPN) statistics was assumed (12). Though a variable cannot be proven to obey the Poisson distribution (at least not in a study of this scope), no evidence was adduced to the contrary in the course of these investigations.

A few peroral infection studies have been conducted with the attenuated oral poliomyelitis vaccine; the statistical approach in these was not stated explicitly (10). However, it has been suggested that the infecting efficiency of the poliovirus was impaired by attenuation (8). This appears to be an after-the-fact assertion based on no direct experimental evidence, but an experimental test of the hypothesis would require administration of virulent virus and would essentially rule out the use of human subjects. We chose swine and their homologous enteroviruses as our experimental system because swine of known background could be obtained locally, whereas most experimental primates (if not embargoed as endangered species) are captured in the wild in tropical countries and have an extremely varied virologic history by the time they can be brought to an American laboratory. A great advantage of using porcine enteroviruses in these studies is that the swine enteroviruses do not infect human or primate cells and are therefore no threat to the personnel handling the virulent viruses or the infected swine.

Research with swine did pose some problems that we had not anticipated. We had intended to get conventionally reared pigs of approximately 3½ weeks of age, acclimate them to the experimental isolators, and then challenge them. Conventional rearing, especially nursing the sow at birth to get the colostrum, seemed important because some evidence exists that pigs deprived of colostrum are immunologically impaired (13). Enteroviruses abound among swine in the Midwest (14), and though the same may not hold true in more arid areas of the United States, we found that virus-free swine were very hard to obtain at 3½ weeks of age, even from farms that appeared to practice quite rigorous sanitation. Furthermore, pigs were not always available at 2 to 8 days of age from farms that we knew practiced good sanitation. When such pigs could be gotten, they did not always survive in our fostering unit to an age where they could be used experimentally. We received a great deal of veterinary consultation when our pigs were dying, but this proved to be of little use with regard to diagnosis, treatment, or prevention. The use of the fostering unit proved valuable in that we no longer had to conduct our experiments with pigs that would later be found to have been infected with virus before they were challenged. It would have been very helpful if the protective effects of bovine colostrum had come to our attention sooner, but the popular report we cite on this subject did not appear until June, 1978 (9).

Our original estimate of the OID for porcine enterovirus 3 (PE3) was based on the observations that no animals became infected after single doses of 100 or 250 PFU (runs III and VIII, respectively), but that two of six animals challenged with 1000 PFU (run V) became infected. As explained earlier, the most probable number of OID's in a given dose can be calculated as the negative Napierian logarithm of the proportion of recipient animals which does not become infected (12). The proportion of animals that did not become infected upon ingesting 1000 PFU of PE3 in run V was 0.67, which indicates that this virus dose was 0.4 OID, and that the OID is 2500 PFU for PE3. This is a small number of observations on which to base the estimate; but run XII, in which two of six pigs became infected when challenged with 1000 PFU of PE3 after eating half their breakfast, appears to support it. A dose of 0.1 OID (250 PFU, in this case) should have a 0.9 probability of not infecting the recipient. However, if a host receives this dose on four successive occasions, the cumulated probability of not becoming infected becomes $(0.9)^4$, or approximately 0.66. Of 11 pigs challenged this way in runs XIV and XVI, six became infected; so the portion that did not was 0.45. This does not differ significantly from the prediction that was based on an estimated OID of 2500 PFU for PE3. But if the results were pooled for all experimental runs in which the total dose of PE3 was 1000 PFU (viz., runs V, XII, XIV, and XVI), and if a new estimate were made based on the findings that 10 out of 23 challenged animals became infected, the new OID estimate would be 1800. The exponential relationship of dose to response in the Poisson formula is such that only order-of-magnitude differences need be taken seriously: 1800 and 2500 PFU are not very different quantities of virus. On the other hand, the OID estimate for PE7, which is 600 to 750 PFU based on runs X, XI, and XV, may well be significantly lower. Because we did not count virus particles in this study, it is hard to be sure of the reason that PE7 appears to cause infection more efficiently than PE3. It is possible that the number of viral particles per OID is the same for both viruses and that PE7 seems more efficient in causing peroral infections only because it is less efficient in infecting tissue cultures (i.e., if there are more particles of PE7 than

of PE3 per PFU, the number of particles per OID may be the same even though more PFU of PE3 are required.

Though we did not undertake a count of virus particles, we believe that the challenge suspensions were essentially monodisperse. Tests of the virus preparation used in early runs showed no significant loss of titer upon filtration through a 50-nm porosity polycarbonate filter. Thereafter, each dose was filtered at 50 nm porosity at the time of challenge. Aggregated virus would perhaps have been more representative of natural conditions, but we were unable to produce a stably aggregated suspension with which to do comparative tests.

We did not choose to assume at the outset of the study that cell cultures were more sensitive than the whole host organism to enteroviruses, even though there were indications in the literature that this was so (10). Now that yet another manifestation of a difference has been seen, we would like to understand the basis of the difference. Within reasonable limits, litter-to-litter, farm-to-farm, and perhaps individual variations (excluding immunity) seem to exert little influence, nor does the use of pigs with full or empty stomachs. The difference between the two porcine enteroviruses may well have been significant, but we cannot tell whether two strains of a single serotype would have differed as much in efficiency as these two, which happened to be of dissimilar serotypes. The effects of extremely early exposure to virus, colostrum deprivation, and challenge by gavage to a pristine stomach remain to be demonstrated. If these conditions are important, our small-scale trial (run XVIII) did not show it. Although the evidence from our study is somewhat limited, it seems to indicate that the OID for enterovirus ingested with drinking water is virus-dependent and host-independent, at least in the absence of antibody against the virus. Unfortunately, our efforts to measure the animals' antibody responses to infection yielded erratic results. The absence of passively acquired antibody to the experimental virus before challenge was general, but active antibody responses were difficult to demonstrate within 2 weeks. Because testing for virus in the stool provided a straightforward basis for determining infection, tests for serologic response were not included in the last few runs. We had hoped that providing each animal with colostrum would guarantee a prompt, active antibody response; but there is some doubt that this is relevant. In any case, ingested antibody may not provide even local protection in the intestine to animals 4 to 6 weeks old. Contents of stomach and intestine from sacrificed control pigs were able to reactivate poliovirus that had been neutralized with coproantibody (15): This result indicated that ingested antibody in the lumen of the digestive tract is likely to afford very limited protection.

Animals held beyond the 2-week observation period and exposed to further large quantities of the same virus provided antisera for use in virus identification. Also, antibody against PE3, labeled with fluorescein isothiocyanate, was used to locate virus in frozen sections of intestine from infected pigs and of infected ileal explant cultures (16,17). Virus antigen appeared to be located in the lamina propria and not in the epithelium of the villi. This finding suggested that the cells the virus would infect were not directly exposed in the intestinal lumen, and it might partly explain the inefficiency with which virus infection occurs upon ingestion (J. Jensen

Kostenbader and D. O. Cliver, unpublished data, 1977). Alternate observations have been reported, however (17).

The effort to determine the OPD was quite unsuccessful. Although both viruses were able to cause paralysis in colostrum-deprived, germfree pigs (2; E. H. Bohl, Ohio Agricultural Research and Development Station, Wooster, 1976), none of the animals infected under the conditions of our experiments became ill. Passage of PE3 in the brain of a pig did not have any perceptible effect on pathogenicity via the digestive tract. Our hopes in this regard smack of Lysenkoism and probably do not have a well-founded theoretical or experimental basis. Neither was dose demonstrably a factor. The 2.5×10^7 PFU of PE3 administered to each animal in run VII could be the equivalent of ingesting at least 2.5 g of feces from an infected animal (18). If pathogenesis required a dose larger than this, enteroviral illnesses would be even rarer than they are. It seems reasonable to infer that even virulent virus can cause disease upon ingestion only if the host organism is being insulted in some other way at the time. We are now doing some exploratory experiments on this subject which we hope will lead to another project.

The irrevocability of chlorine inactivation was not as neatly demonstrated as we had hoped. The dose per animal, had it not been for the chlorine treatment in run XVII, was 1.8×10^6 PFU of PE3 per pig. If virus that was inactivated by chlorine in the sense that it could no longer infect a cell culture were still infectious per os, as has been suggested (19), all five of the challenged pigs could have been infected. Instead, one of the five became infected, which suggested that a fraction of an OID was present in the inoculum. Residual infectivity was detectable in tissue cultures, but not at a level that could be measured by the plaque technique. It does not seem reasonable to attribute this result to the presence of aggregates in the suspension, inasmuch as the suspension had been passed through a 50-nm filter membrane just before the chlorine treatment. The most conservative explanation seems to be that chlorine inactivation is irrevocable and that this single infection was a chance result at this extremely low level of virus infectivity. Further studies in this area are needed.

Our results appear to show that ingestion with drinking water (and, perhaps, food) is not a very efficient way for enterovirus to initiate infection in vivo. Assuming that an infected individual might shed the virus at approximately 10^6 PFU per gram of feces and taking 10^3 PFU as a value intermediate between the two experimentally-determined OID's, the occurrence of a common source outbreak (one in which several persons are ill at the same time) would require that the vehicle be contaminated to a degree that each susceptible consumer would ingest the equivalent of at least 1 mg of feces. This level of contamination is conceivable in food mishandled during final preparation, but it seems unlikely in public water supplies, except in instances of gross post-treatment contamination during distribution of finished water.

On the other hand, an isolated enterovirus infection (and perhaps illness) might occur in a consumer of drinking water contaminated at levels far below that detectable by the most powerful concentration methods available (10,20). Furthermore, this hypothetical infection might give rise to others

by direct (contact) transmission. However, contact transmission appears to be the most prevalent (and perhaps least understood) mode of transfer for enterovirus infections (21); and the vast majority of virus donors in instances of contact transmission have undoubtedly been infected by contact with a previously infected person, rather than by ingestion of virus in water. Thus a susceptible member of a community is probably at greater risk of contracting an infection directly from a family member, a neighbor, or an infected visitor from another community than from a community water supply hypothetically contaminated with virus that has escaped inactivation as the virus level in the treated water approached zero asymptotically. An infected member of a community is a far greater threat to his contacts than to those to whom his infection could be transmitted only via the water route (21). This fact is important because a community has limited resources for preventing virus transmission. It would be detrimental to the public health for the community to dissipate its resources on extraordinary measures in drinking water surveillance and treatment that go beyond the relative hazard that drinking water represents. We believe that our findings provide the beginning of a new basis for judging relative hazards.

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